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# Adaptive radiation of *P. fluorescens* SBW25 in experimental microcosms provides an understanding of the evolutionary ecology and molecular biology of A-L interface biofilm-formation

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## Abstract (200 / 200 words)

Combined experimental evolutionary and molecular biology approaches have been used to investigate the adaptive radiation of *Pseudomonas fluorescens* SBW25 in static microcosms leading to the colonisation of the air-liquid interface by biofilm–forming mutants such as the Wrinkly Spreader. In these microcosms, the ecosystem engineering of the early wild-type colonists establish the niche space for subsequent WS evolution and colonisation. Random WS mutations occurring in the developing population that de-regulate diguanylate cyclases and c-*di*-GMP homeostasis result in cellulose-based biofilms at the air-liquid interface. These structures allow Wrinkly Spreaders to intercept $O_2$ diffusing into the liquid column and limit the growth of competitors lower down. As the biofilm matures, competition increasingly occurs between WS lineages, and niche divergence within the biofilm may support further diversification before system failure when the structure finally sinks. A combination of pleiotropic and epistasis effects, as well as secondary mutations, may explain variations in WS phenotype and fitness. Understanding how mutations subvert regulatory networks to express intrinsic genome potential and key innovations providing a selective advantage in novel environments is key to understanding the versatility of bacteria, and how selection and ecological opportunity can rapidly lead to substantive changes in phenotype and in community structure and function.


**One sentence summary :** (29 / 30 words) The *Pf.* SBW25 experimental system has revealed the evolutionary dynamics and molecular biology of the adaptive biofilm-forming Wrinkly Spreader, providing an insight into bacterial adaptability, radiation and competitive fitness.


**Keywords :** Adaptive radiation, biofilms, competitive fitness, ecological opportunity, intrinsic potential, key innovation.


# Introduction

Experimental studies of microbial evolution have been used to investigate adaptive radiation, a key element in the development of ecological diversity within expanding lineages, and ultimately, in the creation of new species (see Schluter (2000) and reviews by, e.g. MacLean 2005; Buckling *et al*. 2009; Losos and Mahler, 2010; Dettman *et al.* 2012; Bailey and Bataillon 2016; Dykhuizen 2016). In particular, the use of bacterial populations in simple laboratory microcosms has allowed the rapid establishment of links between evolutionary dynamics and the molecular biology underlying adaptive genotypes and key innovations (i.e. changes in phenotype that facilitate improvement in fitness).

One very successful experimental system uses the soil and plant-associated pseudomonad *P. fluorescens* SBW25 (Rainey and Bailey, 1996) in static liquid microcosms where it gives rise to adaptive Wrinkly Spreader (WS) mutants that colonise the air-liquid (A-L) interface through the formation of a cellulose matrix-based biofilm (the key innovation). The nature of WS mutations, phenotype and fitness can now be explained by integrating evolutionary dynamics with an understanding of the underlying molecular biology (reviewed by Spiers, 2014), and involves a deterministic connection between mutations that subvert regulatory systems to induce biofilm–formation, and more stochastic fitness measurements based on population dynamics sensitive to environmental conditions and initial conditions (see **Table 1** for the key challenges and opportunities for *Pf.* SBW25 colonisation of the A-L interface in static microcosms). We believe there are key insights that can be drawn from this model system with links to fundamental microbial biology often overlooked by molecular biologists, relevant to our understanding of the versatility of bacteria and their ability to colonise new environments in the context of pathogenicity, natural and engineered microbial communities.

## Radiation in static microcosms and the colonisation of the air-liquid interface

The adaptive radiation of *Pf.* SBW25 has been studied using microcosms (small glass vials) containing nutrient-rich King's B growth medium which are typically initiated with a founding population of ~$10^4$ cells and incubated over three to five days under static conditions (Rainey and Travisano 1998; Spiers *et al.* 2002; Green *et al*. 2011). During this period the population increases to ~$10^{10}$ cells, and evidence for radiation or diversification can be seen in the appearance of mutant genotypes distinguishable through altered colony morphologies (for this reason they are often also referred to as morphotypes). The establishment of such diversity within the developing population is influenced by spatial structure, nutrients and patterns of physical disturbance (environmental heterogeneity or grains), resource competition and productivity (e.g. Rainey and Travisano 1998; Buckling *et al.* 2000; Kassen *et al.* 2004; Buckling *et al.* 2007; Venail *et al.* 2011; Armitage 2015). One class of mutants, known as the Wrinkly Spreaders (**Figure 1**), also shows an altered niche preference when re-introduced to static microcosms where they colonise the A-L interface through the formation of robust biofilms, in contrast to the wild-type or ancestral *Pf.* SBW25 which grows throughout the liquid column (see Ferguson *et al.* 2013 for a description of another biofilm–forming morphotype known as the Fuzzy Spreaders). The Wrinkly Spreaders have a competitive fitness advantage when at low frequencies compared to the numerically dominant non-biofilm–forming competitors in static microcosms, but not in shaken microcosms where biofilms cannot form or on

agar plates where the Wrinkly Spreader (WS) phenotype is a costly disadvantage (e.g. Rainey and Travisano 1998; Spiers *et al.* 2002; Spiers 2007; Green *et al*. 2011; McDonald *et al.* 2011; Lind *et al.* 2015).

Whilst most biofilm research is focussed on the formation of liquid-solid surface (L-S) interface biofilms in flow cells or micro-titre plates, the ability to produce A-L interface biofilms in static microcosms is common amongst environmental *Pseudomonas* spp., including water and soil isolates, plant-associated and plant pathogenic strains, mushroom pathogens, and psychrotrophic pseudomonads recovered from spoilt meat (Ude *et al*. 2006; Koza 2011; Nielsen *et al*. 2011; Robertson *et al*. 2013) as well as in the opportunistic human pathogen *P. aeruginosa* (Friedman and Kolter 2004). Other examples of A-L interface biofilm–formation may exist where staining of biofilm material attached to vial walls has been measured but where no description of growth over the liquid surface is provided, e.g. in microtitre plates or Calgary biofilm devices in which it is difficult to view biofilms *in situ* (A-L interface biofilms are sometimes also referred to as pellicles, but see the opinion piece by Moshynets and Spiers 2016). Although substantial variation has been observed amongst pseudomonad A-L interface biofilms, they can be categorised into classes and types (Ude *et al*. 2006; Robertson *et al*. 2013), and further differentiated using a combined biofilm assay measuring biofilm strength, attachment levels and total microcosm growth (Robertson *et al*. 2013). This approach, alongside fitness measurements and assays quantifying additional aspects of the WS phenotype, (collectively known as wrinkeality), demonstrate significant WS variation (**Figure 2**) and suggests that Wrinkly Spreaders arise through mutation of a number of different loci that are linked to a common pathway which establishes the WS phenotype *sensu stricto* (i.e. a wrinkled colony morphology on plates and biofilm-formation in static microcosms) (e.g. MacLean *et al.* 2004; McDonald *et al.* 2009; Green *et al.* 2011; Lind *et al.* 2015; Udall *et al*. 2015).

A key insight we draw from this section is that developing populations have the potential to diversify and produce adaptive genotypes that might out-compete the original colonists or colonise new niches. This has significance in the development of infections, natural and engineered microbial communities, where genotypes may change over time effecting host-pathogen interactions, community structure and function.

## Underlying molecular biology of the Wrinkly Spreader

The WS phenotype results from mutations in genes expressing proteins involved in the homeostasis of the intracellular signalling compound c-*di*-GMP, with mutations commonly found in the methylesterase WspF subunit of the chemosensory signal-transduction–like Wsp system that lead to the activation of the associated diguanylate cyclase (DGC) WspR, increased levels of c-*di*-GMP and

the expression of cellulose required for the WS biofilm (**Figure 3 A&B**) (Spiers *et al.* 2002; Spiers *et al.* 2003; Bantinaki *et al*. 2007; McDonald *et al*. 2009; McDonald *et al*. 2011; Udall *et al*. 2015). For example, in the archetypal Wrinkly Spreader the *wspF* mutation is a single nucleotide A – C transition which results in a serine – arginine change at position 301 of the protein (*wspF* S310A). This mutant subunit is predicted to show reduced methylesterase activity based on the crystal structure of the homologous CheB from *Salmonella typhimurium* (West *et al.* 1995) that results in the de-repression of WspR and the synthesis of c-*di*-GMP (Bantinaki *et al.* 2007). Allele exchange experiments swapping *wspF* mutations in Wrinkly Spreaders to the wild-type sequence, and vice versa, have demonstrated that these are sufficient for the WS phenotype and fitness (Bantinaki *et al.* 2007).

Increased levels of c-*di*-GMP lead to the over-expression of partially-acetylated cellulose through the allosteric activation of the cellulose synthase complex (Spiers *et al.* 2002; Spiers *et al.* 2003). Although cellulose expression is common amongst pseudomonads and other bacteria (Ude *et al*. 2006; Nielsen *et al*. 2011; Robertson *et al*. 2013; Arrebola *et al.* 2015; reviewed by Spiers *et al.* 2013; Römling and Galperin 2015), the modification of this polymer by *Pf.* SBW25 using alginate acetylation–like subunits is rare and has only been reported for several phytopathogenic pseudomonads including *P. syringae* pv *tomato* DC3000 and the distantly-related *Bordetella avium* 197N (Arrebola *et al*. 2015; McLaughlin *et al*. 2017). Cellulose is the primary matrix component of the WS biofilm, although a Congo red-binding attachment factor induced by high c-*di*-GMP levels and lipopolysaccharide (LPS) are also required for the WS phenotype (Spiers *et al.* 2002; Spiers *et al*. 2003; Spiers and Rainey 2005). The WS attachment factor has been genetically identified as PGA or PNAG (poly-beta-1,6-N-acety-D-glucosamine) encoded by PFLU0143 – 0146 (Gehrig, 2005; Lind *et al.* 2015), though attachment may also involve amyloid fibrils encoded by the conserved *fapA-F* genes identified in the genome of *Pf.* SBW25 (PFLU2701 – 2696) and a range of other pseudomonads (Dueholm *et al.* 2013).

The hydrophobicity of either PGA or fibrils would allow WS cells and the biofilm matrix to break the A-L interface, suspending the biofilm from above and attaching the periphery of the biofilm directly to the vial walls (after de Jong *et al*. 2009). Like many other biofilms, the WS biofilm is likely to be chemically complex with multiple extracellular polymeric substances (EPS) including cellulose and PGA, LPS, appendages such as pili and flagella, as well as cell debris, all contributing to biofilm strength and attachment (Spiers and Rainey 2005). For example, our recent investigations of biofilm samples has identified extracellular DNA (eDNA) in line with previous observations of *P. aeruginosa* PA01 biofilms (Whitechurch *et al.* 2002) and a homologue of the major outer membrane porin OrpF (PFLU4612) from *P. aeruginosa* PA01 which affects cell surface properties and adhesive capabilities and is linked to c-*di*-GMP regulation (Bouffartigues *et al.* 2015) (Olena Moshynets, Airat Kayumov, Svitlana Rymar and Andrew Spiers, unpublished observations).

The *Pf.* SBW25 c-*di*-GMP regulatory network is likely to be complex as 39 putative DGCs including WspR have been identified in the genome (Silby *et al.* 2009), and a combination of c-*di*-GMP, transcriptional and metabolic systems probably control the expression of cellulose on plant surfaces under natural conditions (Gal *et al.* 2003; Giddens *et al.* 2007; Huang *et al.* 2007b). However, in static microcosms mutations occurring in only a few DGCs or related genes appear to be able to act independently to produce sufficiently high levels of c-*di*-GMP required for the WS phenotype (Bantinaki *et al.* 2007; McDonald *et al*. 2009; McDonald *et al*. 2011; Lind *et al*. 2015; Lind *et al.*, 2017). There are striking similarities between the small suite of DGCs or related genes which lead to the WS phenotype in *Pf.* SBW25 and those producing small colony variant (SCV) morphologies in *P. aeruginosa* isolates from Cystic fibrosis lungs (Smith *et al*. 2006; Malone *et al*. 2012; Malone 2015), and that in *P. aeruginosa* PA01, the overproduction of Pel and Psl EPS is also associated with mutations in *wspF* and increased c-*di*-GMP levels (Starkey *et al.* 2009).

The WS phenotype-activating mutations are examples of adaptive mutations activating intrinsic genome potential resulting in the expression of a key innovation (i.e. biofilm–formation allowing the colonisation of the A-L interface; here we use 'genome potential' to refers to sequences that provide some functionality when expressed under certain circumstances, but which could be expressed in under different conditions where that function or a modification of that function might provide a novel advantage). Although key innovations might arise through the creation of new genes *de novo* or through duplication and divergence of existing sequences (i.e. the innovation–amplification–divergence model), the re-deployment of existing pathways through disruption of regulatory systems allows phenotype divergence and fitness increases to occur more readily and with greater impact (Behe 2010; Andersson *et al*. 2015).

A key insight we draw from this section is that regulatory systems can be subverted by random mutations to activate extant but unexpressed or otherwise–repressed pathways and express complex adaptive phenotypes. This has significance in pathogenicity and the exploitation of natural and engineered microbial communities, where substantive phenotype changes may cause problems in treatment and community structure and function, or provide new opportunities in processing and production.

## Ecosystem engineering and the creation of niche space

The static microcosm initially represents an unstructured or homogeneous environment for colonisation, with a uniform $O_2$ concentration down the liquid column (Koza *et al.* 2011) (**Figure 3 C**). However, this is rapidly degraded by the metabolic activity of the first *Pf.* SBW25 colonists which

establish an $O_2$ gradient within hours that differentiates the microcosms into an $O_2$-rich layer ~200 µm deep at the top of the liquid column and an $O_2$-depleted zone below. The ecosystem engineering of the colonists and the radiation of the population as it develops provides both an ecological opportunity (in the form of a new niche space) as well as the adaptive Wrinkly Spreaders who are able to exploit this modification of the environment (**Figure 3 D&E**) (similarly, *Pf.* SBW25 also modifies the growth medium to which subsequent genotypes adapt, Callahan *et al*. 2014). Ecological opportunity and adaptive radiation are interlinked and include growth and selection feedback mechanisms, as the parameters of the new niche space and the requirements of the adaptive genotype need to be well-matched for successful colonisation (Losos and Mahler, 2010; Yoder *et al.* 2010; Odling-Smee *et al.* 2013; Matthews *et al.*, 2014; Steenackers *et al.* 2016). Changes which effect $O_2$ and nutrient levels, or the physical dimensions of the microcosm, all impact on WS fitness and confirm the link between the $O_2$-rich niche and the WS adaptive genotype (Koza *et al.* 2011; Kuśmierska and Spiers 2016).

The competitive advantage of the Wrinkly Spreader compared to non-biofilm–forming genotypes is negative frequency-dependent (e.g. Rainey and Travisano 1998; Meyer and Kassen 2007). The basis for Wrinkly Spreader success appears to be the rapid domination of the A-L interface by a thin biofilm that intercepts $O_2$ diffusion into the liquid column and limits the growth of other competitors lower down (nutrient levels are comparatively high in King's B microcosms and only begins to limit growth when diluted to very low levels) (Koza *et al.* 2011; Kuśmierska and Spiers 2016). Access to high levels of $O_2$ alters cellular physiology and allows increased growth, final population sizes and biofilm thickness (Spiers et al. 2003; Huang *et al.* 2007b; Koza *et al.* 2011; Kuśmierska and Spiers 2016), and at an early stage of biofilm–formation, most competitive interactions are between the thin layer of Wrinkly Spreaders and the larger non-biofilm–forming population. The adaptive radiation of *Pf.* SBW25 follows the parapatric niche divergence of the high-$O_2$ layer from the lower region which becomes progressively $O_2$-depleted, though there is no physical barrier to migration between these two sections of the microcosm (**Figure 3 D&E**). The Wrinkly Spreaders have a significant impact on this niche divergence, as shallower $O_2$ gradients are formed by populations lacking Wrinkly Spreaders (Loudon *et al*. 2016).

As the WS biofilm matures and deepens, it too divides into a physically-structured upper high-$O_2$ layer and a lower $O_2$-depeleted region. During this period competition increasingly occurs between diversifying WS lineages rather that between Wrinkly Spreaders and non-biofilm–forming competitors. This situation is reminiscent of the Red Queen hypothesis (Liow *et al.* 2011) in which constant competition and adaptation is required for continued Wrinkly Spreader success. This may be mediated or modified by a variety of other evolutionary processes operating within the static microcosms. Kin selection may help develop physically or metabolically-defined niche spaces where cell dispersal is limited (West *et al.* 2006), and an ancestor's inhibition effect may also where parental

cells are suffocated by layers of daughter cells growing above them (Xavier and Foster 2007). Furthermore, the continued development of the biofilm will be effected by the increasing number of cheaters no longer contributing to the construction or maintenance of the biofilm (e.g. Rainey and Rainey 2003; Brockhurst *et al*. 2006; Brockhurst 2007). The development of environmental heterogeneity and genotype diversification in these static microcosms (**Figure 3 D&E**) is predicted by dissipative systems theory where $O_2$ supply is effectively considered a free energy gradient (Loudon *et al*. 2016), and the complexity of the biofilm community will continue to develop until limited by resources or by physical disturbance causing the structure to sink (this event can be considered a systems failure despite the fact that King's B microcosms have sufficient nutrients to allow the development of a second-generation biofilm if allowed, Spiers *et al*. 2003).

A key insight we draw from this section is that populations change local conditions which may favour the development of adaptive genotypes, and such cycles of change and selection are the basis of ecological succession. This has significance in pathogenicity, especially in chronic infections and gastro-intestinal tract disorders, as well as in natural and engineered microbial communities, where the original consortia may be invaded by new members that alter community structure and function.

## Influence of the environment on adaptive radiation

The linkage between ecological opportunity and adaptive radiation suggest that WS evolution, wrinkeality and fitness should all be sensitive to environmental conditions. Indeed, the diversification of *Pf.* SBW25 populations and the maintenance of diversity is effected by structure, physical disturbance, and resources including $O_2$ and nutrients, and variation in WS fitness has been observed within different collections of isolates (e.g. Buckling *et al.* 2000; Kassen *et al.* 2004; Bantinaki *et al.* 2007; Koza *et al.* 2011; Lind *et al.* 2015; Armitage 2015; Kuśmierska and Spiers 2016). Manipulation of physical parameters including A-L interface surface area – volume ratios and the presence or absence of the high-$O_2$ meniscus 'trap' all impact on WS biofilm–formation and fitness (Kuśmierska and Spiers 2016), whilst a comparison of Wrinkly Spreaders isolated from static microcosms and glass bead columns has demonstrated differences in wrinkeality and fitness attributable to origin (Udall *et al.* 2015).

However, correlations between WS phenotype and fitness are poor, suggesting that measurements of microcosm growth, biofilm strength and attachment levels may not effectively capture those aspects of the WS phenotype selected for in static King's B microcosms which also explain competitive fitness advantages (Udall *et al.* 2015). Furthermore, our attempts to differentiate between twenty-four Wrinkly Spreaders on the basis of wild-type or mutant *wspF* alleles (Bantinaki *et al.* 2007; McDonald *et al*., 2011) using phenotype data we have since collected has not proved successful (Andrew Spiers,

unpublished observations), and this suggests that the WS genotype to phenotype (G-P) map is likely to be equally difficult to establish.

Phenotypic variation is not random but is regulated by internal and external factors (Sharov 2014). Although allele replacement experiments have confirmed the importance of mutations in DGCs or related genes for the WS phenotype and fitness (Bantinaki *et al.* 2007; McDonald *et al.* 2009), internal factors such as antagonistic pleiotropic (and epistasis) effects may differ between Wrinkly Spreader mutations and produce variation within the WS phenotype *sensu stricto*. The multiple DGCs identified in the *Pf.* SBW25 genome suggests the complex and dynamic regulation of c-*di*-GMP homeostasis, with functional DGC redundancy upstream and c-*di*-GMP–sensitive pleiotropy downstream. Perturbation of c-*di*-GMP homeostasis may lead to variation in substrate utilisation patterns and fitness changes (MacLean and Bell 2003; MacLean *et al.* 2004), and the archetypal Wrinkly Spreader *wspF* S310A mutation results in proteomic changes in metabolic pathways not linked with the WS phenotype that might nonetheless be associated with fitness-reducing effects (Knight *et al.* 2006). Although homeostasis may appear to restrict phenotypic variation, the mutation of complex regulatory networks allows the adjustment and multi-tasking of functions, and the establishment of new connections between regulatory components and functions which may result in diversifying phenotypic effects (Sharov 2014). Additional mutations outwith these networks will add further phenotypic complexity, and in Wrinkly Spreaders isolated from aging or multiple-transfer populations such secondary mutations may ameliorate the antagonistic pleiotropic effects of the initial WS mutation, or add more elements to the developing WS phenotype.

A key insight we draw from this section is that small changes in initial conditions can have a big impact on subsequent population growth and diversification, and on the phenotype and success of any adaptive lineages that may appear. This is a central tenant of Chaos theory (the ‘butterfly’ effect), and has significance in natural and engineered microbial communities where the complexity of interactions will restrict the predictability of adaptive radiation.

## Alternative routes to the colonisation of the A-L interface by biofilm-formation

Despite the competitive success of the Wrinkly Spreader in diversifying population of *Pf.* SBW25 in static microcosms, the intrinsic genome potential exploited by this class of adaptive mutants is not the only means by which the A-L interface can be colonised. *Pf.* SBW25 is known to produce at least five different biofilms which can be differentiated by mutation, biofilm matrix components and phenotype. These include the true Wrinkly Spreaders and the Viscous mass (VM) biofilm produced by wild-type

*Pf.* SBW25 when induced with $FeCl_3$ (Koza *et al.* 2009) which utilise cellulose as the primary biofilm matrix, WS-like mutants derived from cellulose-deficient (Δ*wss*) strains including CBFS 2.1 (Gehrig, 2005) and the PWS mutants that use PGA instead (Lind *et al.* 2017), disrupted LPS–associated Fuzzy Spreaders (FS) (Ferguson *et al.* 2013), and matrix-independent cell–chaining (CC) phenotypes (Lind *et al.* 2017).

Comparison of WS, VM and CBFS 2.1 biofilms including quantitative measurements of biofilm strength, attachment levels, and rheology, plus measurements of competitive fitness including the ability to invade a larger population when numerically rare, clearly differentiate these structures and their ecological success in static microcosms, with the WS biofilm being the most robust and providing the greatest fitness benefit in pair-wise competitions (Koza 2011; Anna Koza and Andrew Spiers, unpublished observations). Similarly, fitness and invasion assays have been used to differentiate WS, FS, PWS and CC mutants (Rainey and Travisano 1998; Ferguson *et al.* 2013; Lind *et al*. 2017).

These different routes to the colonisation of the A-L interface by *Pf.* SBW25 is an example of evolutionary convergence and underscores the strong selection in static microcosms for access to $O_2$. Significantly, mutation of three key DGCs or associated regulators result in the expression of cellulose or PGA through the disruption of c-*di*-GMP homeostasis, and if these genes are deleted, there are a further thirteen mutational pathways that will still activate the WS or WS-like phenotype (Bantinaki *et al.* 2007; McDonald *et al.* 2009; McDonald *et al.* 2011; Lind *et al.* 2015; Lind *et al*. 2017). It would appear that the pleiotropic effects associated with mutations altering c-*di*-GMP homeostasis and the expression of cellulose and PGA collectively determine the fitness cost to biofilm–formation, whereas the growth advantage offered by access to higher $O_2$ levels provides the fitness benefit in colonising the A-L interface in static microcosms (MacLean *et al.* 2004; Koza 2011; Lind *et al*. 2017).

A key insight we draw from this section is that where there is sufficiently strong selection, multiple mutational pathways may be used to activate unexpressed or otherwise–repressed genome potential in order to allow bacteria to exploit new ecological opportunities with subtly differing phenotypes determined by pleiotropic effects. This has significance in pathogenicity, as isolates producing similar symptoms may have significantly different responses to pharmaceutical treatments such as antibiotics.

## Concluding comment

The use of simple experimental microcosms to investigate adaptive radiation and the ecological success associated with complex phenotypes is often regarded by microbiologists as having little

relevance to the colonisation of natural environments by bacteria and the functioning of the communities they establish, or indeed, of the value of such approaches to assess the evolutionary or ecological significance of particular pathways of interest. However, we believe that the key insights we have drawn from this model system have relevance in a range of areas, including pathogenicity, especially in the treatment of chronic infections and long-term gastro-intestinal disorders where both pathogen populations and host communities will change over time and with medical intervention, and in natural and engineered communities such as those used for biocontrol, bioremediation, and biotechnology processes to convert biomass, produce chemicals or energy, where communities and key members will also change in response to environmental conditions. In each of these, bacteria should be seen as being enormously adaptable and able to rapidly access intrinsic genome potential through simple mutations. As populations grow, they will modify ecosystems, diversify and adapt, and this will drive ecological succession and change community functions in a manner not predictable if bacteria are considered to be cellular automatons with limited and unchanging response to abiotic and biotic factors.

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533

534 **Figures, figure legends and Table 1 are included in separate files.**

535 Figure 1. The adaptive Wrinkly Spreader genotype.

536 Figure 2. Wrinkly Spreader isolates show considerable variation in wrinkeality.

537 Figure 3. Elements of adaptive radiation in static microcosms.

538 Table 1. Challenges and opportunities for adaptive radiation of *Pf.* SBW25 in static microcosms
539 and the colonisation of the A-L interface by biofilm–formation.

# Figure legends

Figure 1. **The adaptive Wrinkly Spreader genotype.** When incubated in static microcosms, wild-type *Pf.* SBW25 grows throughout the liquid column (left microcosm) and produces rounded and smooth colonies on agar plates. In contrast, the Wrinkly Spreader colonises the A-L interface by forming a robust biofilm demonstrating a change in niche preference (right microcosm) and produces wrinkled colonies. Image : A. Spiers.

Figure 2. **Wrinkly Spreader isolates show considerable variation in wrinkeality.** The combined biofilm assay can be used to determine quantitative differences in WS phenotypes, collectively known as wrinkleality. Shown here are the mean ± standard errors (ovals) for biofilm strength (grams / $OD_{600}$) versus attachment levels ($A_{570}$ / $OD_{600}$) for 12 independently-isolated Wrinkly Spreaders recovered from static microcosms (data are adjusted for growth using $OD_{600}$ measurements). There are significant differences in strength ( $p = 0.01$) and attachment ($p < 0.01$) as determined by ANOVA. However, growth and attachment do not have a significant effect on biofilm strength ($p > 0.05$) when modelled using a GLM approach and are not sufficient to predict the robustness of WS biofilms. Raw data were from Udall *et al*. (2015); microcosm growth is determined by optical density measurements after vigorous mixing ($OD_{600}$), biofilm strength is determined using small glass balls (grams), and attachment levels determined using Crystal violet staining and absorbance measurements ($A_{570}$); see this reference for further details.

Figure 3. **Elements of adaptive radiation in static microcosms.** Random mutations activate intrinsic genome potential to produce key innovations. (A) The *Pf.* SBW25 genome encodes the seven-gene chemosensory signal-transduction–like Wsp system and the ten-gene Wss cellulose synthase operon (*wspF* is indicated by the black rectangle). (B) The Wsp complex (grey oval) is inactive when *Pf.* SBW25 is growing in static microcosms, but mutations disrupting the regulatory role of the WspF subunit (black circle) in many Wrinkly Spreader isolates results in the production of c-*di*-GMP (double hexagons) by the DGC WspR (grey circle). Increased levels of c-*di*-GMP then induce the cellulose synthase complex (large grey circle) to express cellulose (black wiggly line) and attachment factor (not shown) required for the WS biofilm or key innovation. (C) The early colonists of static microcosms are ecosystem engineers and initially experience an unstructured environment (i) with uniform $O_2$ levels down the liquid column (indicated by the vertical dashed line). However, their metabolic activity establishes an increasingly acute $O_2$ gradient (dashed then solid black lines) which stratifies the liquid column into a

high-$O_2$ zone (ii) and an $O_2$-depleted region underneath (iii). (D) The diversifying population drives parapatric niche divergence in static microcosms to create new niches and support adaptive lineages. The initial niche (white circle) is transformed into a high-$O_2$ niche (grey bulge) colonised by the first biofilm–forming Wrinkly Spreaders (i) and an $O_2$-depleted niche that continues to support the ancestral genotype. As the WS biofilm matures, the $O_2$-depleted niche is further degraded, whilst additional niches (black bulge) may develop within biofilm structure to support new genotypes (ii). As these niches are not separated physically, genotypes can migrate from one to another, though as bacteria are non-sexual (and in this case not able to support horizontal gene transfer), hybridisation does not occur. (E) The diversification of the population established by the colonists (black dot at the start of the time-line going from the left to right) can also be mapped onto the creation and divergence of niches. A critical mutation (white dot) generates the first Wrinkly Spreader lineage able to colonise the high-$O_2$ niche (indicated here as crossing the dashed line and corresponding to D (i) above). Further diversification of the Wrinkly Spreaders (or other genotypes) leads to new adaptive genotypes able to colonise additional niches developing within the biofilm structure (indicated as crossing the dotted line and corresponding to D (ii) above).

Table 1. Challenges and opportunities for adaptive radiation of *Pf.* SBW25 in static microcosms and the colonisation of the A-L interface by biofilm–formation.

| | |
|---|---|
| Initial conditions | *Evolvability of the ancestor* : *Pf.* SBW25 has intrinsic genome potential : a complex c-*di*-GMP regulatory system with multiple DGCs linked to the expression of EPS that can be used as biofilm matrix components<br><br>*Limiting factors in the environment* : $O_2$ is the primary resource restricting growth rate and final population sizes in static microcosms. Cells are subject to constant movement by random diffusion and micro-currents within the liquid column and microcosms are subject to random physical disturbance (sufficient to dislodge and sink biofilms).<br><br>*Potential for adaptation* : Overcoming limiting factors to achieve faster growth rates and higher final population sizes. |
| Ecological opportunity | *Ecosystem engineering* : Colonists change the initial environment by establishing an $O_2$ gradient in which flux through the A-L interface is balanced by uptake by individuals in the liquid column. This creates a high-$O_2$ niche space at the top of the liquid column available for colonisation.<br><br>*Parapatric niche divergence* : Conditions in both niches develop as the biofilm matures and populations continue to diversify. $O_2$ will be further depleted in the liquid column whilst the $O_2$-rich region at the top will become shallower as the biofilm matures. The developing biofilm will provide physical structure and increased metabolic activity which may influence cell distributions, nutrient and waste diffusion. |
| Fitness concerns | *Physical structure* : The biofilm secures access to high–$O_2$ levels by retaining cells at the A-L advantage interface in a cost-effective manner. If costs increase, WS fitness will be reduced.<br><br>*Competitors* : Establishment of the biofilm reduces $O_2$ available to competitors lower down in the liquid column, restricting growth rate and final population sizes. WS fitness is initially high when competition is largely between Wrinkly Spreaders and non-biofilm–forming genotypes, but decreases as Wrinkly Spreaders begin to dominate numerically. |
| Future developments | *Increased systems complexity* : Wrinkly Spreader competition within the biofilm, continued population diversification and complexity niche divergence, will add multiple niches defined by physical space and metabolic opportunities.<br><br>*System collapse* : Random physical disturbance generally causes biofilms to sink within 5 – 7 days, and although biofilm-formation may be re-initiated, physical disturbance and nutrient levels will ultimately determine system productivity. |

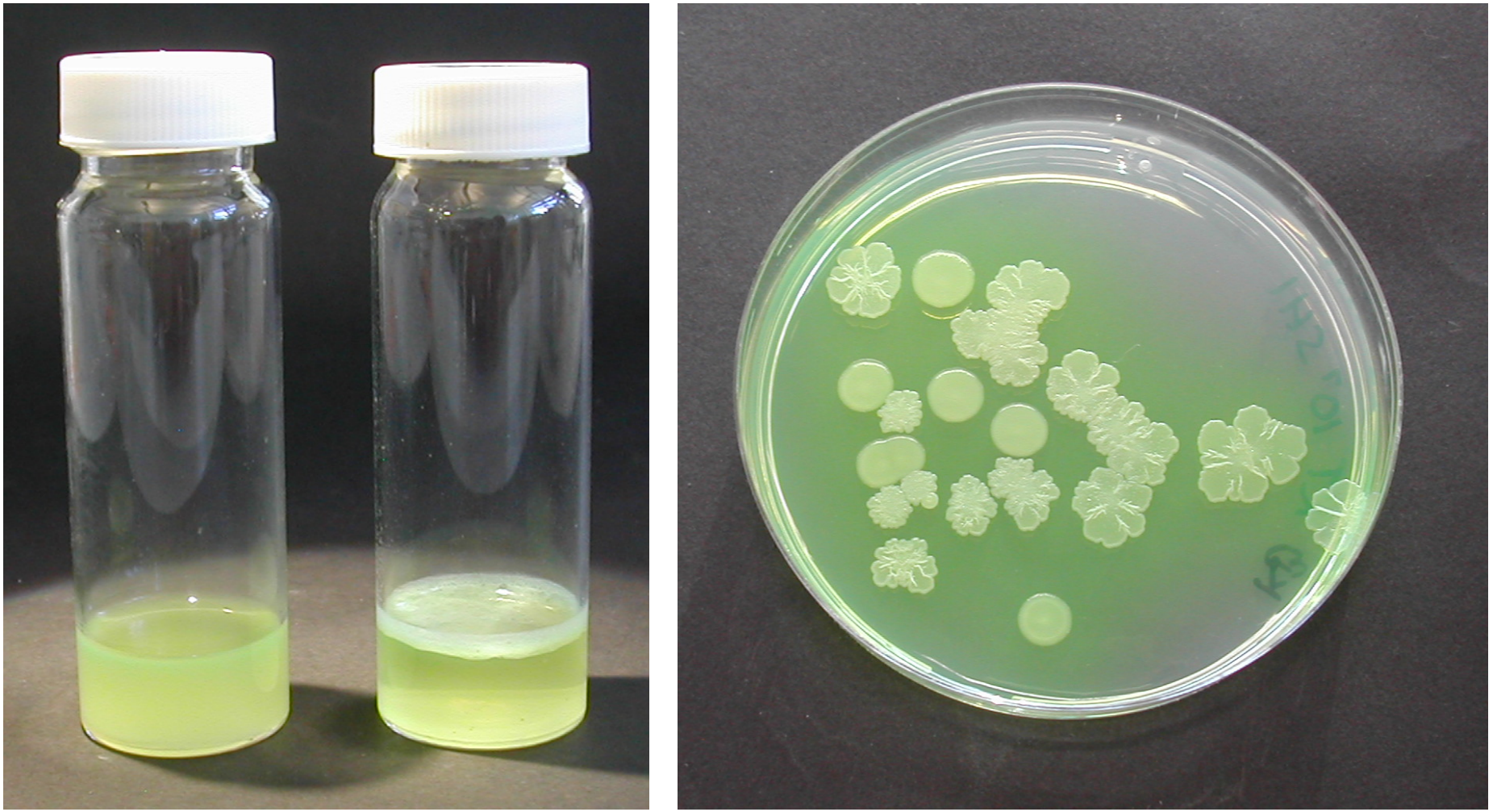


Figure 1

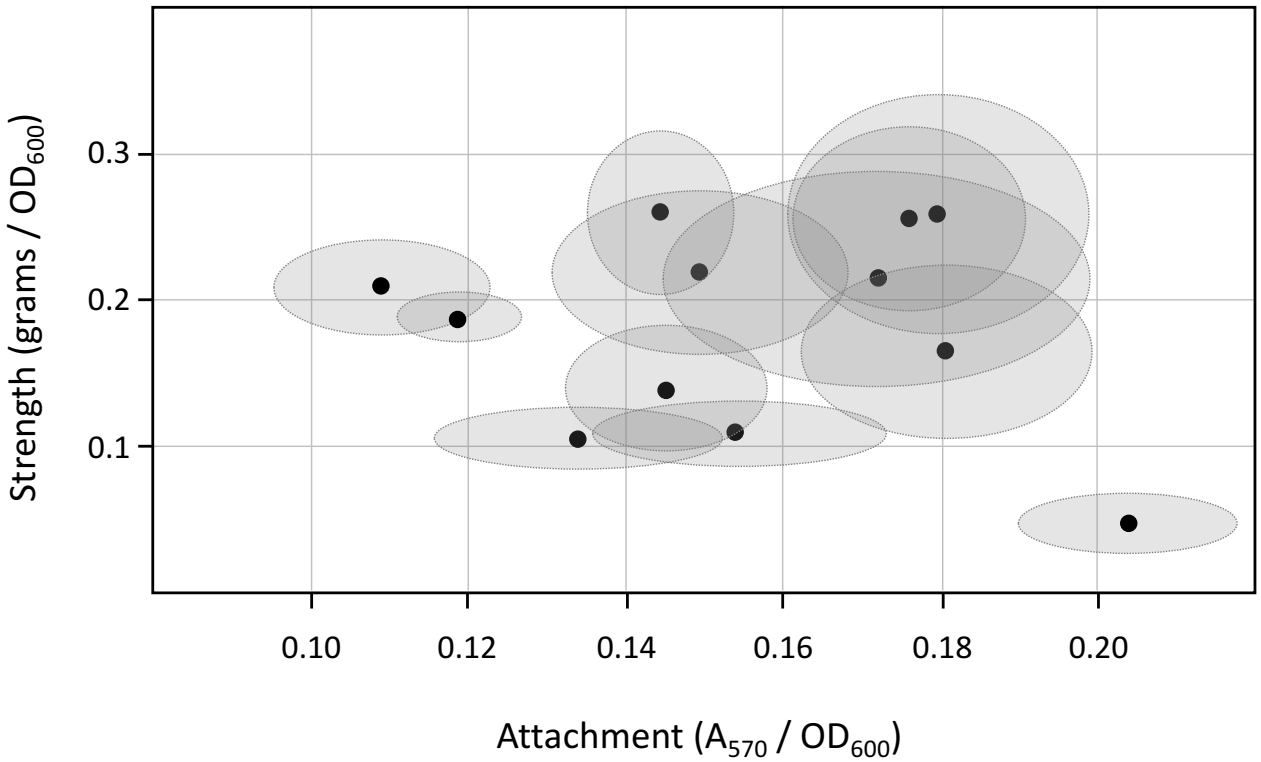
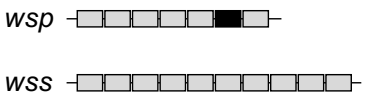
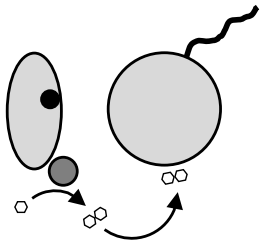

Strength (grams / OD600)
0.3
0.2
0.1
0.10
0.12
0.14
0.16
0.18
0.20
Attachment (A570 / OD600)

Figure 2

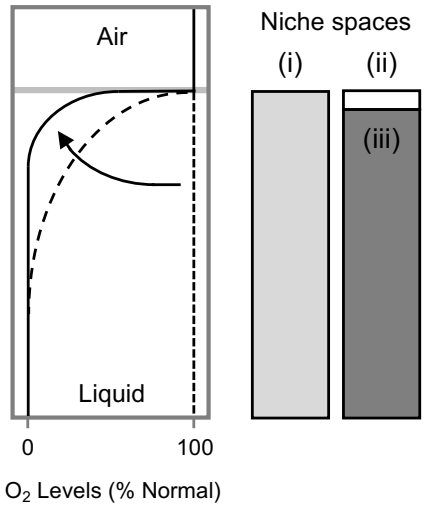
(A) Genome potential



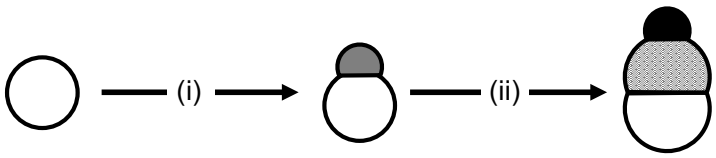

(B) C-*di*-GMP induction



(C) Ecosystem engineering




(D) Parapatric niche divergence




(E) Genotype divergence